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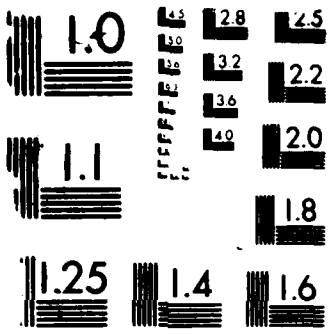

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We have increased the amount of 16S ribosomal RNA (rRNA) sequence data that can be gathered by dideoxynucleotide-terminated sequencing from rRNA-specific primers with reverse transcriptase. We have compiled phylogenetically useful, partial or complete sequences of the 16S rRNA from about 220 organisms and organelles. An aligned collection of published 16S rRNA sequences is available in printed form or in several electronically accessible forms. The 16S rRNA data were used to infer the relationships among the archaebacteria, and of the archaebacteria to the eubacteria and eukaryotes. Our programs for phylogenetic tree inference are available for use in the VAX/VMX operating system environment. We examined sources of systematic error in the inference of molecular phylogenies and concluded that lineage-to-lineage variations in the rate of accumulating mutations in the rRNA gene can lead to significant errors, which can be decreased by using models of sequence evolution that acknowledge that all sites in the molecule are not equally mutable.

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Use of 16S Ribosomal RNA Sequences to Infer Relationships among Archaebacteria

ANNUAL REPORT

Goals

a) Use the phylogeny of 16S ribosomal RNA (rRNA) sequences to determine the evolutionary relationships among archaebacteria.

b) Insure that 16S rRNA sequence data for the best-characterized archaebacterial species are available to provide an overall phylogenetic framework within which other species can be placed.

c) Maximize the reliability of the inferred phylogenies and define the limitations of these phylogenies.

d) Actively work with other investigators to produce 16S rRNA sequence data from the archaebacteria of greatest interest to them so that a unified body of data will accumulate relating many diverse archaebacteria.

Accomplishments

(numbers in text refer to the listing of publications and presentations below)

Because of the general applicability of the sequencing, data collection, and data analysis techniques which form the core of our ONR funded project, there is substantial synergism between the ONR project and the other phylogenetic and natural microbial populations work in the laboratory. In order to fully reflect the progress on the ONR project, portions of the overlapping work are included below.

Rapid rRNA sequencing methodology

The techniques for rapidly determining partial 16S rRNA sequences by dideoxynucleotide-terminated sequencing from "universal" 16S-rRNA-specific primers with reverse transcriptase have been refined to yield more data per molecule (in particular by decreasing the number of sequence positions of ambiguous identity). The current protocol will appear in a forthcoming volume of Methods in Enzymology devoted to cyanobacteria (10). In addition, we are approximately three-fourths through the writing of a comprehensive "laboratory manual" which explains all of the procedures (from RNA isolation through phylogenetic analysis) at a level appropriate for non-molecular biologists.

Sequencing efforts in the laboratory have focused on the accumulation of partial sequences of 16S rRNAs from members of two eubacterial groups: the sulfur-oxidizing bacteria (in collaboration with Arthur Harrison, University of Missouri, Columbia) and the cyanobacteria (14, 17). Also, we have taught the rapid rRNA sequencing technique to, and have collaborated with, Michael Ghiselin (California Academy of Science), Rudolf Raff (Indiana University), Elizabeth Raff (Indiana University), and Marilyn Milberger (Craig Nelson laboratory, Indiana University) for the purpose of inferring the relationships among the metazoan phyla (5, 11, 15, 20). Several additional investigators have visited our laboratory for assistance in learning to do rapid rRNA sequencing: Dan Distel (Scripps Institute of Oceanography), to study sulfur-oxidizing symbionts of clams (13); Reinhardt Rossen (Institute for Great Lakes Research), to study lake microbiology; Peggy Romero (Howard Gest laboratory, Indiana University), to study an unusual group of photosynthetic bacteria; Farooq Azam and Michelle Pontius-Brewer (both from Scripps Institute of Oceanography), to study ocean microbiology; Jed Fuhrman (State University of New York, Stony Brook), to study ocean microbiology; Colleen Cavanaugh (Harvard Unversity), to study sulfur- and methane-oxidizing symbionts of marine invertebrates; and Tineke Burger-Wiersma (University of Amsterdam), to study the free-living prochlorophyte, Prochlolothrix hollandica. We have also collaborated with Paul Romaniuk (University of Victoria, British Columbia, Canada) on a phylogenetic analysis of the genus Campylobacter (8).

One reason for accumulating 16S rRNA sequences from diverse organisms is to provide a data base for use in phylogenetically "identifying" rRNA genes which are isolated directly from the DNA present in the biomass of a natural population (3, 4, 9, 19). The rRNA gene characterizations provide an overview of the component organisms in the population, without requiring laboratory cultivation of the organisms. We had previously isolated (as recombinant DNAs) the rRNA genes from the microbial community of a 91°C hot spring (Octopus Hot Spring in Yellowstone National Park). Three rRNA genes (two eubacterial and one archaebacterial) from this population have now been partially sequenced and are nearly ready for phylogenetic analysis. We have also assisted David Ward (Montana State University, Bozeman), who is using these techniques to characterize the microbial mat communities associated with Octopus Hot Spring.

Analysis of archaebacterial phylogeny

Approximately 220 phylogenetically useful, partial or complete 16S rRNA sequences from a wide variety of organisms and organelles have been compiled. Analyses of the sequence relationships within the archaebacteria and the relationships of the archaebacteria to other organisms have been published (1, 2, 16, 18). The analyses render untenable the suggestions of Lake and colleagues (Lake et al., 1985) that the eubacteria derive from photosynthetic archaebacteria (halobacteria). Instead, our analyses support a view in which the archaebacteria form a distinct (holophyletic) group. (See below for a discussion of alternative approaches to the sequence data analysis.)

Availability of analysis programs

We have written a detailed description of our tree inference method for a Methods in Enzymology volume on ribosomes (7). Our phylogenetic tree inference programs have been improved so that it is possible to examine

systematically the best alternatives to the "optimal tree." We are providing copies of our sequence analysis and tree inference programs (which are dependent upon the VAX/VMS computing environment) to several institutions: the University of Illinois, Urbana; Montana State University, Bozeman; the University of Victoria; National Jewish Center for Immunology and Respiratory Medicine, Denver; the State University of New York, Stony Brook; the Dana-Farber Cancer Research Center, Boston; and Kings College, London.

Availability of 16S rRNA sequence data

The published 16S rRNA sequences in our data collection are available either individually or in aligned form. We can provide them in a printed copy, on nine-track tape, by dial-up connection to our MicroVAX (running the VMS operating system), or by BITNET electronic mail. The format of the aligned sequences is a text file, arranged similarly to a published sequence alignment. The nucleotides are supplied in IUB recommended representation, and the alignment gaps are represented by hyphens. Minor changes of format could be made to accomodate other needs.

Potential systematic errors in phylogenetic tree inference

We have investigated the potential for systematic errors in the phylogenies inferred from rRNA sequences resulting from disparate rates of mutation acceptance (different average "molecular clock" rates). The potential for error in the inferred phylogenetic trees can be substantially decreased by utilizing a more realistic model of sequence evolution which acknowledges that all sites in the 16S rRNA are not equally mutable (18). Specifically, we have examined the effect of assuming that the relative substitution rates across the 16S rRNA fits a log-normal distribution function. For the approximately 950 positions that we routinely analyze, the empirically determined width of the distribution is such that 95% of the sequence positions change at rates between 1/8'th and 8 times the median rate of change. Although initial studies were based upon the relationships of mitochondrial rRNAs, the observations have proven to very general. Other groups in which lineage-to-lineage differences in the rate of fixed mutation accumulation potentially influence the accurate inference of phylogenetic relationships include archaebacteria (archaebacterial rRNAs have evolved more slowly than the rRNAs of one or both other kingdoms, and among the archaebacteria there are also substantial variations) (1), echinoderms (5, 11), major eubacterial divisions, and chloroplasts in relation to cyanobacteria (14, 17). When we apply this alternative data treatment to the investigation of the relationships of archaebacteria with eukaryotes and eubacteria, we arrive at the same answer as we have in the past: the archaebacteria are distinct from these two other groups.

Comparing the sensitivity of various tree inference methods to statistical error

There has been significant controversy regarding the "correct" method of analysis of sequence data. We have taken initial steps toward a quantitative analysis of the various techniques. We originally chose a distance matrix method of phylogenetic tree inference because Schwartz and Dayhoff (1978) had presented evidence that it is statistically superior to parsimony-type analyses, and Felsenstein (1978) had demonstrated a significant source of

systematic error intrinsic to parsimony-based analyses. Because there appear to be few citations of the Schwartz and Dayhoff conclusion, we have performed similar, but more exhaustive, simulations of phylogeny reconstruction by parsimony, a distance method, and, also, cluster analysis. These studies have led us to essentially the same conclusions as Schwartz and Dayhoff, although the magnitude of the superiority of the distance method is much less for nucleotide sequences than for the amino acid sequence data considered by the previous studies.

Alternative analysis methods applied to archaebacterial phylogeny

Wolters et al. (1986) have argued that a proper cladistic analysis of 16S rRNA sequences reveals a specific relationship between eukaryotes and thermophilic (sulfur-dependent) archaebacteria, to the exclusion of the eubacteria, methanogens and halophilic archaebacteria. By restricting their analysis to slowly varying sequence positions (conveniently identified by their lack of variation within well-estabished groups, i.e. positions that are conserved among eukaryotic rRNAs and conserved among eubacterial rRNAs) they limited the analysis to data for which parsimony-based methods should be appropriate. Wolters et al. note three 16S rRNA sequence positions (1303, 1334 and 1408 in the Escherichia coli sequence) at which eukaryotic and thermophilic archaebacterial rRNAs share a common nucleotide, while the other sequences share a different nucleotide. Thus, these three positions are most parsimoniously explained by a specific eukaryote/thermophilic-archaebacteria relationship, a view previously expressed by Lake et al. (1984). However, it is not clear how these authors can ignore the analogous nucleotide usage patterns at positions 338, 367, 393, 923, 973, 1211, and 1393 (E. coli position numbers) which all specifically relate the eukaryotes to the eubacteria, and relate all the archaebacteria to one another. Thus, the balance of the evidence in a parsimony analysis of slowly changing 16S rRNA sequence positions is that the archaebacteria are a group. If one similarly considers the proposal of Lake et al. (1985) that the eubacteria are specifically related to the halophilic archaebacteria, then there are additional slowly changing positions in conflict: 33, 332, 551, 939, 1074, 1083, and 1344. Thus, when used with the most slowly varying sequence positions in the molecule, those at which the method should be most reliable, a parsimony analysis gives the same interkingdom relationships as the distance matrix analysis.

Lake (1987) has argued that none of the above analysis methods adequately account for events in the peripheral branches of a phylogenetic tree that can mimic the early events which defined that actual branching order, and he has proposed an analysis technique, "evolutionary parsimony," that is intended to rectify the potential problem. In particular, the technique seeks to statistically eliminate any tendancy for combinations of transversion type mutations in peripheral tree branches to be confused with transversions in the central branch of a four organism, unrooted phylogenetic tree. The inference of the correct branching order is then an issue of how many sequence positions have undergone a single transversion (actually any odd number would do) mutation in the central branch of the tree and no changes at the same positions in any of the peripheral branches. Rapidly changing positions will not contribute useful information since they will almost certainly have undergone one or more changes in the peripheral branches (which, in a

multikingdom phylogeny, span billions of years). When we apply Lake's analysis technique to the most slowly changing sequence positions (those that display no intragroup variation within the eukaryotes or within the eubacteria) we arrive at the same conclusions as we do with the distance matrix analyses: the archaebacteria are a united group, distinct from the eubacteria and eukaryotes.

Plans for the Next Year

a) Complete the "lab manual" for the rapid sequencing of rRNAs.

b) Expand the manual to include the isolation and sequencing of 16S rRNA genes from samples of natural populations.

c) Effort will be made to provide our compilation and alignment of 16S rRNA sequence data in additional data formats (unfortunately, standardization of formats is poor for alignments of multiple sequences).

d) Survey the authors of previously published 16S rRNA sequences for published and unpublished revisions to the sequences. There has been a tendancy for such revisions to appear (when they appear at all) in contexts where they can easily be overlooked.

e) Cooperate with the University of Wisconsin Genetics Computer Group to integrate phylogenetic tree inference programs into the package of programs which they distribute. As an initial step, we have agreed to assist them in implementing our programs on the University of Wisconsin campus.

f) Transfer our phylogenetic tree inference programs to a supercomputer (most likely the Cray XMP at the University of Illinois National Center for Supercomputing Applications).

g) Continue development of phylogenetic tree inference methods which are less sensitive to known sources of systematic and random error. Particular attention is being given to dealing with site-to-site and lineage-to-lineage variations in the mutation accepance rate.

Recent Publications

1. Woese, C.R., and Olsen, G.J. (1986). Archaebacterial Phylogeny: Perspectives on the Urkingdoms. Syst. Appl. Microbiol. 7, 161-177. Also reprinted in Archaebacteria '85, O. Kandler and W. Zillig (Eds.). Stutgart and New York: Gustav Fischer Verlag, pp. 161-177.

2. Pace, N.R., Olsen, G.J., and Woese, C.R. (1986). Ribosomal RNA Phylogeny and the Primary Lines of Evolutionary Descent. Cell 45, 325-326 (mini-review).

3. Pace, N.R., Stahl, D.A., Lane, D.J., and Olsen, G.J. (1986). The Analysis of Natural Microbial Populations by Ribosomal RNA Sequences. Adv. Microbial Ecol. 9, 1-55.

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5. Raff, R.A., Anstrom, J.A., Chin, J.E., Field, K.G., Ghiselin, M.T., Lane, D.J., Olsen, G.J., Pace, N.R., Parks, A.L., and Raff, E.C. (1987). Molecular and Developmental Correlates of Macroevolution. In Development as an Evolutionary Process, R.A. Raff and E.C. Raff (Eds.). New York: A.R. Liss, pp. 109-138.

6. Stahl, D.A., Lane, D.J., Olsen, G.J., Heller, D.J., Schmidt, T.M., and Pace, N.R. (1987). A Phylogenetic Analysis of certain Sulfur-Oxidizing and related Morphologically Conspicuous Bacteria by 5S Ribosomal RNA Sequences. Internat. J. Syst. Bacteriol., in press.

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10. Lane, D.J., Field, K.G., Olsen, G.J., and Pace, N.R. (1987). Reverse Transcriptase Sequencing of rRNA for Phylogenetic Analysis. Meth. Enzymol., in press.

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12. Karl, D.M., Taylor, G.T., Novitsky, J.A., Jannasch, H.W., Wirsen, C.O., Pace, N.R., Lane, D.J., Olsen, G.J., and Giovannoni, S.J. (1987). A Microbiological Study of Guaymas Basis High Temperature Hydrothermal Vents. In preparation.

13. Distel, D.L., Giovannoni, S.J., Lane, D.J., Olsen, G.J., Pace, B., Pace, N.R., Stahl, D.A., and Felbeck, H. (1987). Sulfur-Oxidizing Symbionts: Analysis of symbiont phylogeny, specificity, and origins by 16S ribosomal RNA sequences. In preparation.

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16. Olsen, G.J. (1987). The Earliest Phylogenetic Branchings: Comparing rRNA-based evolutionary trees inferred with various techniques. Cold Spring Harbor Symp. Quant. Biol. In preparation.

Presentations of this work were given at the following meetings:

17. 86th Annual Meeting of the American Society for Microbiology, Washington, D.C., March 23-28, 1986.

18. Macromolecules, Genes, and Computers (organized by Dana-Farber Cancer Research Institute), Waterville Valley, New Hampshire, August 11-17, 1986.

19. Fourth International Symposium on Microbial Ecology, Ljubljana, Yugoslavia, August 24-29, 1986 (two talks).

20. American Society of Zoologists Annual Meeting, Nashville, TN, December 27-30, 1986.

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Lake, J.A., Henderson, E., Oakes, M., and Clark, M.W. (1984). Proc. Natl. Acad. Sci. USA 81, 3786-3790.

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